Mechanisms of Membrane Repair and the Novel Role of Oral Phospholipids (Lipid Replacement Therapy®) and Antioxidants to Improve Membrane Function.

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If the fundamental biological maxim– 'structure subserves function' – remains paramount, the evolutionary commitment to generating, managing and maintaining the vast array of lipids required by humans to survive and prosper has presented science with a complex task to fully elucidate our lipid repertoire and determine their biological functions. Arguably the most important of these lipids are the phospholipids that are the mainstay of all cellular membranes. The wide variety of cellular and organelle membranes and the existence of special membrane lipid regions and domains allows for the design of specific lipid replacement therapies to support and maintain the structure and function of cellular membranes. The authors discuss some of the biological processes and evolving strategies related to lipid replacement therapy and its use along with antioxidants for the constitutive management of mitochondrial and other cellular membranes as well as the functional gains from the utilisation of lipid replacement to improve cellular membranes biological functions.

Overview

During the last century, the structure of the cell membrane has evolved, from a vague boundary between the cell's interior and exterior to a complex, dynamic bilayer of phospholipid molecules with intercalated proteins and glycoproteins. Although most scientific research has primarily focused on the role that membrane proteins and glycoproteins play in cell recognition, signalling, transport and association with other structures inside and outside cells, a growing body of research points to the sweeping and subtle roles that membrane phospholipid molecules play in biology and medicine.

There have been many articles published during this time that have described components of cellular membranes and their organisation and function, but the following authors have provided significant landmarks in the structure, physiology and functionality of cellular membranes.

In 1925 **Gorter and Grendel** [1] proposed on the basis of cell lipid analyses that the cell membrane has just enough lipids to be surrounded by an opposing bilayer of phospholipids. Further work by **Davson and Danielli**[2] in 1935 proposed that cell membrane was made up of a phospholipid bilayer with the heads of the lipids facing outward and sandwiched between two layers of proteins. This structure was later visualised by **J. D. Robertson** [3] with the use of electron microscopy.

He also proposed that membranes were symmetrical structures.

Then in 1972 **Singer and Nicolson** [4] proposed the hypothesis that the cell or plasma membrane is actually a discontinuous two-dimensional liquid bilayer matrix of phospholipids, with amphipathic globular proteins and glycoproteins that are intercalated to various degrees into the lipid bilayer and can diffuse or move laterally in the plane of the membrane. In this model was the proposal that the bulk of the phospholipids were able to freely diffuse in the membrane plane, whereas a small fraction specifically interacted with membrane proteins. This fundamentally changed the prevailing perspective that the cell membrane was a fixed and 'passive diffusion barrier' to being understood as a dynamic structure, but also one that follows restrictions imposed by thermodynamics. They also provided evidence for the first time that cell membranes were asymmetric between their inner and outer surfaces a factor that contributes to the curvature stress in membranes and also that the characteristics of the lipid matrix depend upon the physical properties of the individual lipid components in the membrane. [5]

By the late 1970s **Bretscher** [6] and others were exploring explanations in addition to the thermodynamic restrictions proposed by **Singer and Nicolson** [4] to define mechanisms for the persistence of asymmetry. Concepts such as enzymatic driven responses by ATP- dependent flippases began to gain traction as asymmetric manipulators in both the inner and outer leaflets of the bilayer, with key phospholipids, such as phosphatidylserine (PS), cardiolipin and others, being highly sequestered in the inner portion or leaflet of the membrane. [6] Lipid flippases help to maintain membrane lipid asymmetry, and in eukaryotes (*all organisms except for bacteria and archaea are eukaryotes*) they are also intimately involved in membrane budding and vesicle trafficking. The ATP-dependent flippases are members of well-characterised protein families, whose other members transport non-lipid substrates across cell membranes. [7]

For example, PS is a primary membrane phospholipid that tends to be sequestered in the inner leaflet of the plasma membrane. Any changes in the asymmetric distribution of PS can lead to various biological consequences. PS (*and other phospholipids*) asymmetric distribution may also play a role in apoptotic signalling and associated immunogenic responses.

Over the last 40 years, researchers have shown that the outside and inside of membrane lipid bilayers contain vastly different distributions and ratios of various lipid molecules. These structures are constantly changing and can be induced to change by interactions within or outside of cells. Their dynamics along with the dynamics of integral membrane proteins regulate cell signalling, transport, enzyme activity, adhesion, cell shape, structure, and movement, among other properties.

The major structural lipids in eukaryotic membranes are the glycerophospholipids: phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphotidylglycerol (PtdGly), phosphatidylinositol (PtdIns) and phosphatidic acid (PA). Their hydrophobic portion is a diacylglycerol (DAG), which contains saturated or cis-unsaturated fatty acyl chains of varying lengths.

In the 1980s **Karnovsky, Klausner** [8] and others proposed that within cellular membranes there were lipid rafts, also known as microdomains, which are in effect areas, or clusters of lipids of a differing composition

from the rest of the membrane. In some cases these lipid rafts were found to be enriched in cholesterol, sphingolipids (*one of the two dominant 'families' of lipids; a range of phospholipids containing sphingosine e.g. ceramides, sphingomyelins, gangliosides and cerebrosides that occur in high concentrations in the brain and other nerve tissue*) and other differentiated lipids. These lipids could present as a planar product such as a raft or could be invaginated and produce a caveolae, or flask shaped structure rich in proteins and lipids that serve several, albeit as yet not fully elucidated, functions in signal transduction and transport. Thus folates, chemokines, and many pathogens or their secreted products (e.g. HIV, cholera toxin) may also enter cells through membrane caveolae.

Membranes Act as Dynamic Cell Organelles

Membranes act not only as diffusion barriers but also as dynamic cell organelles, contributing to the provision of intracellular mediators, such as arachiodonic acid and inositol phosphates, and also working with integral membrane proteins to modulate various activities. This includes membrane-mediated transport, receptor signalling, enzyme activity, ion channel transfers, cell adhesion and shape and structure modifications through inner membrane surface-cytoskeleton interactions. They are also involved in cellular movement; as seen in T-cell or macrophage transitions and fibroblast motility, as well as transport, defensive and production activities, including phagocytosis, endocytosis and exocytosis.

Membrane Challenges

The essential transport of phospholipids between the two lipid layers of a membranes bilayer is highly dependent on the presence of adenosine triphosphate (ATP). As ATP production declines so does the utilisation of existing membrane- and serum-derived lipids for the purpose of asymmetrical membrane management and composition.

ATP is a primary end product of cellular mitochondrial function, and it is easily diminished if mitochondrial inner membrane (MIM) potential is reduced due to increased mitochondrial outer membrane permeabilisation (MOMP) or oxidation of inner MIM cardiolipin and other lipids. Mitochondrial production of ATP is directly linked to maintenance of MIM transmembrane chemical/electrical potential.

The MIM hosts the most important redox reactions of the cell, converting the energy of nutrients by the oxidation of NADH and succinate and the transfer of the released energy via the electron transport chain (ETC) into MIM electrochemical potential ($\Delta\Psi$), which in turn drives conversion of ADP into ATP. The ETC transfers electrons from NADH (or other substrates) to molecular oxygen in the complex multi-step process termed mitochondrial respiration.

The side effects of the energy production in the MIM include the production of reactive oxygen (ROS) and reactive nitrogen species (RNS). Mitochondria are responsible for some 90% of our body's production of these free radicals, which then act as metabolic signals or destructive factors. [9] Although ROS and RNS have been classically known for their damaging effects, increasing evidence of their importance in regulating and maintaining normal processes in living organisms

has been accumulating. Therefore, the term 'redox regulation' seems to better describe the redox status of mitochondria and its consequences. One of the areas where redox balance is most comprehensively required is in the MIM of mitochondria.

Mitochondria possess several unique characteristics, among which are the presences of mitochondrial DNA (mtDNA), their mode of inheritance – from the maternal germ line only -the dynamic nature of their structure, their indispensable roles in fuel metabolism and energy production, and the established links of mitochondrial dysfunction to various metabolic abnormalities. Therefore, as might be expected, compromised mitochondria can be an underlying mechanism for a myriad of pathological conditions. Most of these conditions present initially as fatigue and a reduction in fatigue resolution with rest, but they also involve many other signs and symptoms that are related to reduced availability of high energy molecules such as ATP.

Mitochondria resemble bacteria in many ways, particularly in their double membrane and DNA structures.[10] Multiple observable similarities have led to the conclusion that mitochondria were once free-living bacteria that became intracellular symbionts that lost most of their DNA and encoded proteins. These similarities also suggest that when mitochondrial molecular patterns are released from cells by cellular membrane trauma, they might activate immunity by mimicking bacterial motifs or patterns. Intrinsic molecular motifs like this are referred to as "damage-associated molecular patterns" (DAMPs) or 'alarmins'. [11]

Polyunsaturated phospholipids are essential for life: they represent the structural core of membranes both as an uninterrupted bilayer and as a microenvironment for transmembrane proteins; they act as precursors of physiological regulators and as a fuel and energy resource [17]

Mitochondrial Permeabilisation

The mitochondrial membrane permeability event MOMP is a decisive event in the functional decline and eventual execution of apoptosis or programmed cell death. It is also causally linked to a decline in bioenergetic function via different mechanisms, not merely due to cytochrome c dispersion. This includes at higher levels the generation of fatigue, and the increased production of DAMPs, such as contained in mtDNA, formyl peptides, cytochrome c and other mitochondrial apoptogenic proteins that bind to key inflammatory promoting receptors, further provoking risk for MIM electrochemical potential decline and resulting reduction in ATP production. [12]

Other processes contributing to mitochondrial dysfunction may be linked to alterations in the phospholipids of the MIM. Several groups have reported pre-apoptosis-associated changes in cardiolipin content, including oxidation, [13] "reorganisation," [14] and even relocation of cardiolipin from the MIM to other membrane compartments. [15]

Mitochondrial membranes such as the MIM are especially sensitive to oxidation. For example, the polyunsaturated nature of the mitochondrial membrane phospholipids makes membranes highly susceptible to peroxidative modifications. One of the most contemporary examples includes selective peroxidation of

cardiolipin in MIM of cells undergoing apoptosis. Cardiolipin is unique to mitochondria and is synthesised from the phospholipids PA, PtdGly and PtdEtn.[16] Cardiolipin peroxidation products are required for the mitochondrial membrane permeabilisation, release of pro-apoptotic factors and completion of the cell death programme. Therefore, the search for effective inhibitors of cardiolipin peroxidation is critical to discovery and development of anti-apoptotic antioxidants.

Repairing Small Amounts of Mitochondrial Membrane Damage

Mitochondria continually produce highly reactive superoxide anions as a by-product of electron transport during oxidative phosphorylation. These ROS and RNS free radicals damage proteins, lipids, and DNA (Box 1). Damage to proteins in the ETC may worsen the situation by stimulating the production of even more ROS.[18] Recent studies have found that autophagy (the programmed destruction of cells) and mitophagy (the programmed destruction of mitochondria) function as major sensors of redox signalling at the interface between cell stress adaptation and cell death. Autophagic activities are mediated by complex molecular machinery, including membrane phospholipids. Dysfunction of autophagy may result in abnormal mitophagy, mitochondrial function and oxidative stress.

Fission & Fusion

Mitochondrial fission and fusion play critical roles in maintaining functional mitochondria when cells experience metabolic or environmental stresses.

Fusion of mitochondria helps mitigate stress by mixing the contents of partially damaged mitochondria with undamaged mitochondria as a form of complementation in which undamaged phospholipids and co-factor nutrients are utilised or re-used to maintain their viability. Mitochondrial fusion remains a largely unknown process, albeit some steps have now been exposed. Legros et al. used a green and a red fluorescent protein targeted to the mitochondrial matrix to demonstrate that mitochondrial fusion occurs in human cells, is efficient and achieves complete mixing of matrix contents within as little as 12 hours.[19] They showed that fusion requires mitochondria to be viable and is mediated by mitofusins. These mitofusions activate the process in which mitochondria of healthy cells undergo fusion. The course of action occurs in three steps;

1) The mitochondria align themselves, end to end;
2) The outer membranes of the two organelles fuse with each other;
3) The inner membranes fuse with each other, thus forming a larger intact mitochondrion.

Mitochondrial fusion therefore, represents a rescue mechanism for impaired mitochondria by the mixing of contents (proteins, lipids and mitochondrial DNA) and the unification of the mitochondrial compartment, permitting it to maintain its functionality and allow it to continue to play roles in cellular development, aging and energy dissipation. [20,21]

Fission of mitochondria is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis (*or mitophagy removal of mitochondria*) during high levels of cellular stress. This is necessary, because defective mitochondria can be toxic by generating excessive amounts of ROS, by consuming high quantities of ATP through reversal of ATP synthase, and by interfering with other metabolic processes.

BOX 1

MITICHONDRIAL STRESS
Various insults can cause damage to mitochondria:
Environmental (radiation, toxic chemicals, dysbiosis, nutrient deficiencies and excess)
Genetic (mutations in genes for metabolic processes or repair pathways)
Spontaneous (ROS generated as by-product of uncoupling of electron transport)
TYPES OF TISSUE DAMAGED:
MtDNA, nuclear DNA
Cellular Proteins
Phospholipids
PROBLEMS CAUSED BY DAMAGE:
Loss of metabolic functions (ATP synthesis, metabolism, etc.)
Excess ROS made by defective mitochondria
F_1F_0-ATPase may, instead of making ATP, consume ATP to help generate inner membrane (MIM) potential
CELLULAR RESPONSES TO DAMAGE:
DNA repair
Proteases
Lipases
Mitochondrial unfolded protein response
Mitophagy
Apoptosis
Autophagy, Fusion and Fission

Managing Mitochondrial Life stages

As stated above, **Fusion** allows mitochondria to compensate for one another's membrane defects by sharing undamaged components and thereby helps maintain energy output in the face of stress - providing that adequate raw materials are made available during the process. It can also mitigate the effects of environmental damage through the exchange of proteins and lipids with other mitochondria. [22] However, when a certain threshold of damage is reached, mitochondria are eliminated wholesale by autophagy (mitophagy). [23]

Fission replicates healthy mitochondria and segregates the most seriously damaged mitochondria to preserve the health of the mitochondrial network in addition to regulating morphology and facilitating mitochondrial trafficking. The highly dynamic mitochondrial fusion and fission cycles are proposed to balance two competing processes: compensation of damage by fusion and elimination of damage by fission (and mitophagy).

Role of Membrane Phospholipids and Lipid Replacement Therapy (LRT®)

What do the lives and functions of mitochondria have to do with the novel oral supplementation of scientifically selected glycophospholipids known as Lipid Replacement Therapy (LRT®)?[24,25] Utilising orally ingested glycophospholipids, antioxidants and other ingredients cellular membranes can be repaired and returned to their normal structures and functions. By carefully controlled packaging in special formulations oxidation-resistant lipids and other compounds can provide suitable membrane-specific lipids in quantities adequate to maintain cellular membrane fluidity and physiologically healthy mitochondrial membrane states. LRT® phospholipids are bound to lipid transport proteins, lipid-binding proteins or lipoproteins, which bind the hydrophobic tails of the lipids into their interiors or hydrophobic regions of their structures, and sequester them away from oxidation compounds or free radicals. Thus, the phospholipid tails are in hydrophobic (*not hydrophilic*) environments due to their hydrophobic structures. This protects the delicate tails of the phospholipids and provides unoxidised polyunsaturated lipids for utilisation in the inner and outer membranes of the cells and mitochondria.

This is also important in mitochondrial fusion, fission and membrane repair as well as providing suitable quenching molecules for ROS and RNS free-radicals and diminishing inappropriate mitochondrial collapse and mitophagy. The functional outcomes of the use of LRT® can be reflected in increased ATP synthesis, decreased membrane permeabilisation, increased inner membrane potential, diminished DAMP production and eventually decreased levels of fatigue and improvements in various organ functions, such as cognition and mood.

Additional improvements in mitochondrial repair have also been noted in diets that are limited in caloric intake without malnutrition and an adequate mineral intake, utilisation of antioxidants such as COQ10,[27] vitamin E,[28] curcumin,[29] resveratrol and rotterlin [30] and other associated or complementing nutrients.

Summary

The reduction of inappropriate mitochondrial and cellular membrane damage can be achieved at four levels:

1) By lowering exposure to environmental pollutants with oxidising properties or free radicals,
2) By increasing levels of endogenous and exogenous antioxidants,
3) By lowering the generation of oxidative stress by stabilising mitochondrial inner membranes and related energy production and efficiency, or
4) By replacing damaged, oxidised membrane lipids with undamaged, polyunsaturated phospholipids.

Importantly, mitochondrial membrane damage cannot be reversed by use of exogenous antioxidants alone—it requires LRT® to replace damaged membrane lipids in order to restore membrane function.

The role of phospholipids in membrane physiological and biological functions, growth development and cell death are as yet incompletely understood. What is clear is that phospholipids are essential and utilised by the highly volatile membranes of cells and especially mitochondria to manage normal cellular energy metabolism and accelerated physiological degradation. Eventually the loss of mitochondrial function is felt as an overall loss of energy and impairment in activities and processes that require cellular energy systems for their function. This loss of mitochondrial function is a common feature in all chronic illnesses and diseases.

In addition, there are many initiators of cellular permeabilisation of the plasma membrane and mitochondrial membranes, which cause a significant component of membrane collapse and activation of processes that contribute to a wide range of chronic illnesses. Oral supplementation of antioxidants may provide some compression of the rate of collapse but it cannot reverse this process, but the concomitant use of oxidation-protected phospholipids (LRT®) further enhances the optimal management of all cellular membranes and supports adequate membrane potential, especially in the MIM to facilitate production of ATP.

There is growing evidence that redox regulators, related active mediators, cellular organelle functions, and surrounding environments are all tied together in intricate networks affecting the whole body energetic status, metabolism, state of health, presence of disease and even lifespan.

The usefulness of LRT® has been demonstrated in in vitro and animal experiments as well as in human clinical trials.[23, 24] LRT® impacts not only mitochondrial function and membrane potential, but also has the potential to reduce age-related cellular damage in human subjects, returning cellular systems and physiological functions to functionalities seen in much younger people. [31]

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